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1 ***Diversity and bioactivity of fungi associated with the***
2 ***marine sea cucumber *Holothuria poli*: disclosing the***
3 ***strains potential for biomedical applications***

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21

22 **Running head:** Bioactive fungi of *Holothuria poli*

23

Abstract

Aims: Identification of the mycobiota associated to the marine echinoderm *Holothuria poli* and investigation of cytotoxic and pro-osteogenic potential of isolated strains.

Methods and results: Fungal strains were isolated from the animals body-wall, intestine, and faeces. The species identification was based on DNA barcoding and morphophysiological observations. Forty-seven species were identified, all Ascomycota and mainly belonging to *Aspergillus* and *Penicillium* genera. Sixteen strains were grown on three media for chemical extraction. Cytotoxic activity was tested on a hepatic cancer cell line (HepG2), the cells viability was evaluated after treatment using a resazurin based assay (AlamarBlue). Pro-osteogenic activity was tested on human Mesenchymal Stem Cell, differentiation was measured as the alkaline phosphatase production through reaction with *p*-nitrophenylphosphate or as the cells ability to mineralize calcium using a colorimetric kit (StanBio). Cytotoxic activity was recorded for four fungal species while five out of 48 extracts highlighted bioactivity toward human mesenchymal stem cells.

Conclusions: The presence of relevant animal-associated mycobiota was observed in *H. poli* and selected strains showed cytotoxic potential and pro-osteogenic activity.

Significance and importance of this study: Our work represents the first report of a Mediterranean sea cucumber mycobiota and highlights the isolates potential to synthesize compounds of pharmaceutical interest for regenerative medicine.

48

49 **Keywords:** Environmental mycology, Pharmaceuticals, Cytotoxicity, Marine
50 Fungi, Sea cucumber, Mediterranean Sea

51

52 **Introduction**

53 Fungi are important actors of the marine ecosystem, studies on the molecular
54 diversity of the micro-eukaryotic community shown that they inhabit most of the
55 marine habitats with disparate ecological roles (Jones and Pang 2012). To
56 date, 1,412 species have been documented in the marine environment (Jones
57 et al. 2019) but information on unexplored habitats and DNA sequencing data
58 collected over the past 20 years identified a possible further 10,000 (Jones
59 2011). To overcome this lack of knowledge and encrypt the real microbial
60 biodiversity existing in the sea, the isolation and identification of fungal
61 communities living in association with new substrates is an essential task.
62 Several studies investigated the mycobiota existing in the Mediterranean Sea,
63 mainly focusing on algae, sponges and seagrasses (Garzoli et al. 2014; Gnani
64 et al. 2017; Bovio et al. 2019), describing particular marine fungal communities
65 living in association with marine organisms. The up to date picture shows over
66 200 fungal species isolated from Mediterranean substrates, with several
67 recurring taxa such as *Aspergillus*, *Penicillium* and *Acremonium*. Common
68 species such as *Penicillium chrysogenum*, *Trichoderma harzianum* and
69 *Cladosporium sphaerospermum* were also identified, but the discovery of key
70 species and trends in marine fungal populations strongly rely on the description
71 of fungal communities from additional substrates.

Beside their ecological value, the description and isolation in pure culture of fungal strains from new marine substrates represent a valuable resource for biotechnological applications for their potentially unusual biochemical properties. Marine derived fungi had shown potential to synthesize pharmaceutical compounds such as anticancer, antibacterial, anti-inflammatory, antiviral, pro-osteogenic and others (Ebel 2012; Prince and Samuel 2015; Silber *et al.* 2016) as well as compounds with cosmeceutical and nutraceutical properties (Imhoff 2016). Marine drug discovery surveys had underestimated the microbial potential in the past, often focusing on bioactive molecules extracted from macro-organism without considering the substrate microbial colonization. This approach led to mistakenly assign the biosynthesis of chemotherapeutic Ecteinascidin to the marine tunicate *Ecteinascidia turbinata* (Rinehart *et al.* 1990) instead of the real producer, its bacterial endosymbiont (Schofield *et al.* 2015). To address the microbe as the metabolite producer instead of an animal or plant is very advantageous: once isolated, a fungus can be grown *in vitro*, allowing further studies with no more expensive and environmental-impacting sampling campaigns. Moreover, the development of fermenters for fungal cultivation allows industrial production and extraction of the metabolite from the biomass (Syed 2019).

Holothuria poli (Delle Chiaje, 1823) is a Mediterranean and oceanic sea cucumber, widely distributed in the Mediterranean Sea as well as in the northern Red Sea and in the Canary Islands Sea (WORMS 2020). It has been intensively studied for its secondary metabolites production, demonstrating a strong antifungal activity (Ismail *et al.* 2008), but never deeply analysed for its associated microbiome. A single study by Omran and Allam (2013) isolated one

strain of *Candida albicans* and a few bacteria associated with specimens collected in Egyptian Mediterranean sea, while a rich mycobiota was recorded by Pivkin (2000) on internal and external organs of three species of sea cucumbers sampled in the Pacific Ocean. A thorough study of the mycobiota associated to *H. poli* is particularly important for the wide distribution of this echinoderm in the Mediterranean area and for its ecological role as filter feeder. Given its remarkable production of antifungal compounds, the associated mycobiota might be specifically selected and contain strains with particular biochemical properties and potential producers of new pharmaceutically bioactive compounds.

In this study we describe the fungal community living in association with *H. poli*. The isolates biotechnological potential was investigated as their ability to synthesize metabolites for pharmaceutical applications. Investigated targets were the cytotoxicity against an hepatic cancer cell line (HepG2) and the bioactivity toward stem cell to promote their differentiation into bone or cartilage cell progenitors (Alves *et al.* 2011; Besio *et al.* 2019a, 2019b).

Materials and methods

***Holoturia poli* collection**

Six individuals of *H. poli*, together with their faeces (rejected sand), were collected in September 2013 along the rocky coast of the Tabarka peninsula (Tunisia). Samples were maintained at 0-4°C during transportation. In order to evaluate the fungal colonization on different animal districts, specimens were firstly washed in sterile seawater, surface sterilized with 70% ethanol and then

underwent surgical manipulation in a sterile condition to separate the body wall (B), intestine (I) and faeces (S).

Isolation and identification of associated fungal strains

Each sample was homogenized using steel beads and MM400 tissue lyzer (Retsch GmbH, Haan, Germany), then was diluted 1:10 w/v in phosphate buffer. An aliquot of each sample was dried to calculate the samples' number of colony forming units *per* gram of dry weight (CFU gdw⁻¹). One ml of suspension was plated on Corn Meal Agar Sea Water (CMASW: 2g corn meal extract, 15g agar dissolved in 1l of sterilized artificial seawater, 2% w/v Sea Salts in ddH₂O) added of an antibiotic mix (Gentamicin 80mg l⁻¹, Piperacillin-Tazobactam 100mg l⁻¹ - Sigma-Aldrich, Saint Louis, USA). Three replicates *per* sample were performed (Panno *et al.* 2013; Gnani *et al.* 2017). A total of 54 plates were incubated at 24°C in the dark up to one month to allow the isolation of all fungi, including the slow-growing ones. Plates at days 2, 7, 14, and 21. Strains from each fungal morphotype and from each matrix (B, I, S) were isolated in axenic culture and preserved at the *Mycotheca Universitatis Taurinensis* (MUT – <http://www.mut.unito.it/en>).

Identification of the isolated fungi was carried out with a polyphasic approach, combining morpho-physiological and molecular methods. Fungi were firstly morphologically classified on the basis of specific taxonomical keys (Seifert *et al.* 2011; Samson *et al.* 2014a, 2014b). Subsequently, molecular analyses were performed by sequencing specific genomic regions (for details see Table S1). Taxonomic assignments were based on similarity to reference sequences

available at GenBank (nBlast; mismatch 1/-2; gap costs linear) and CBS databases. Molecular findings were confirmed morpho-physiologically. Newly generated sequences were deposited in GenBank database (Table S2)

Fungal chemical extraction

Sixteen strains (Tab. 3), encompassing the most representative species isolated from *H. poli*, were cultivated following a preliminary OSMAC (One Strain Many Compounds – Bode *et al.*, 2002) approach. Fungi were cultured on different nutrient conditions to stimulate the production of different metabolites: each strain was inoculated in duplicate (two 9cm Petri dishes for each condition), onto three media containing different carbon and nitrogen sources and/or salinity. Based on pre-trials, media used were: Malt Extract Agar (MEA: agar 20g, glucose 20g, malt extract 20g, peptone 2g, dissolved in 1l ddH₂O), Malt Extract Chloride Agar (MECIA: agar 20g, malt extract 20g, NaCl 17g, dissolved in 1l ddH₂O) and Soy Mannitol Agar (SMA: agar 20g, soy peptone 20g, mannitol 20g, NaCl 15g, dissolved in 1l ddH₂O). Plates were incubated at 24°C for four weeks to allow fungal growth. After incubation, fungal biomass and medium were lyophilized and minced using an Ultraturrax device (IKA, Staufen, Germany), extracted adding 1:10 w/v of solvent mix (1:1 methanol–dichloromethane) and stirred overnight. Suspensions were filtered and dried using Rotavapor (KNF, Freiburg, Germany). Using this procedure 48 crude dry extracts were obtained.

Fungal extracts chemical fingerprinting

Extracts chemical fingerprint was performed by analytical HPLC with a Waters Alliance 2695 (Waters Corporation, Milford, MA) using Luna C18 column (250×10mm id, 5µm Phenomenex Inc., Torrance, USA) coupled to a Diode Array Detector (DAD, Waters 996). The crude extracts were re-suspended at final concentrations of 10mg ml⁻¹ in CH₂Cl₂/MeOH (1:1, v/v), 20µl were injected and eluted with a gradient of H₂O/MeCN/Formic acid (90:10:0.1 to 0:100:0.1 – flow 1ml min⁻¹). Detection was performed by Sedex 55 evaporative light-scattering detector (SEDERE, Alfortville, France), wavelengths set at 214, 254 and 280nm.

Extracts cytotoxicity assessment

To assess the extracts cytotoxicity, a miniaturized high throughput assay was performed using an automated workstation (PerkinElmer, Waltham, USA). A liver cancer cell line (HepG2) was treated with seven extracts concentrations (200pg l⁻¹, 2ng ml⁻¹, 20ng ml⁻¹, 200ng ml⁻¹, 2µg ml⁻¹, 20µg ml⁻¹ and 200µg ml⁻¹): dry extracts were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Saint Louis, USA) and diluted in cell culture medium to reach the working concentrations.

Cells were cultured in Basic Medium (BM) containing: αMEM and 1% penicillin/streptomycin - Thermofisher, Waltham, USA plus 10% FBS – Sigma Aldrich, Saint Louis, USA. Culture flasks were maintained at 37°C, 5% CO₂ in humidified atmosphere. The screening was performed by seeding 5x10³ cells in in each well of flat-bottom 96-well plates, incubated to settle overnight. Subsequently, treatment was performed by adding 100µl of BM with either

diluted extracts or controls, all conditions tested in triplicate. DMSO was kept at 0.5% in the experimental wells. The positive control cells were treated with 0.5% DMSO and negative controls with 10% DMSO to induce a cytotoxicity. Cells were then incubated at 37°C, 5% CO₂ for 72 h.

Cell viability after treatment was evaluated by measuring the cell reduction of the vital reagent resazurin (AlamarBlue - Thermofisher, Waltham, USA). A 10% v/v of reagent was added to the wells and plates incubated for 6 h at 37°C, 5% CO₂ to let the cells metabolize the reagent. After incubation, fluorescence was measured at 531nm excitation wavelength, 572nm emission wavelength, and Lethal Dose 50 (LD₅₀) calculated.

Extracts pro-osteogenic bioactivity

High Throughput Assay Procedure

To assess the extracts pro-osteogenic activity a miniaturized high throughput assay was performed using an automated workstation (PerkinElmer, Waltham, USA). Human Mesenchymal Stem Cells (hMSCs) were isolated from the bone marrow of healthy donors at the Galway University Hospital after informed consent and ethical approval. Cells were cultured in growing medium containing α -minimal essential medium (α -MEM, Life technologies), 10% fetal bovine serum (FBS, Sigma) and 1% penicillin/streptomycin (P/S, Life technologies) supplemented with 1ng ml⁻¹ fibroblast growth factor-2 (FGF-2, Peprotech). Culture flasks were maintained at 37°C, 5% CO₂ in humidified atmosphere. Actively proliferating cells were detached from the flasks and resuspended in Basic Medium for osteogenesis (BM) containing phenol red

free Dulbecco's modified eagle medium low glucose (DMEM-LG, Life Technologies), 10% FBS and 1% P/S. Ten thousand cells were seeded in flat bottom 96-well plates (Sarstedt) and incubated overnight at 37°C, 5% CO₂. To evaluate a promotion of differentiation, cells were treated with fungal extracts diluted in Osteogenic Medium (OM) containing BM with 100nmol l⁻¹ Dexamethasone, 100µmol l⁻¹ Ascorbic acid 2-Phosphate, 10mmol l⁻¹ β-glycerophosphate (Sigma-Aldrich, Saint Louis, USA). Crude extracts were tested at four non-toxic concentrations (based on the cytotoxicity screening results), DMSO was kept at 0.5% in experimental wells and controls. Positive control cells were treated with OM+0.5% DMSO and negative control cells with BM+0.5% DMSO. Cells medium was appropriately refreshed 72 h after treatment and early differentiation marker alkaline phosphatase (ALP) measured seven days after treatment. Cells intracellular ALP level was measured using an enzymatic assay involving the reaction enzyme-substrate (*para*-nitrophenilphosphate) as previously described (Bruder *et al.* 1997).

Osteogenic hits re-screening

Fungal extracts that showed to induce cells ALP expression higher than the set threshold (Ctr+ average + 3SD) were selected as positive hits and re-tested for bioactivity. Extracts were tested at 20µg ml⁻¹ in two media formulations: added to OM to evaluate promotion of differentiation or in Incomplete Osteogenic Medium (IOM: OM lacking β-glycerophosphate) to evaluate their ability to induce differentiation in absence of one essential component for *in-vitro* hMSCs osteogenic differentiation. Cell seeding and treatment was performed as

described before, the ALP level was measured after 7 days differentiation. The cell calcium mineralization was measured as a late differentiation marker after 12 days treatment. Due to the longer incubation time, two additional medium changes were performed at days 7 and 10. The calcium mineralized in the extracellular matrix was solubilized in 1mol l⁻¹ HCl and quantified as previously described (Jaiswal *et al.* 1997) with a StanBio Calcium Liquicolour Kit (StanBio Calcium assay kit - Thermofisher, Waltham, USA).

Statistical analyses

Statistical analyses were performed using PRIMER 7.0 (Plymouth Routines In Multivariate Ecological Research; Clarke and Warwick, 2001). The fungal species biodiversity was evaluated calculating Shannon-Weaver's index (H') and Gini-Simpson's index (1-Lambda). One-way ANOVA and *t*-test on bioactivity results were performed using GraphPad Prism version 7.0 (GraphPad Software, La Jolla, California, USA). One-way ANOVA and Bonferroni post-test (significance level of 0.05) was used to compare the data in the osteogenic assessment and OSMAC effect.

Results

Holothuria poli associated mycobiota

From 18 samples, obtained by three districts of six animals, 498 fungal strains belonging to 17 taxa were obtained. The retrieved isolates were ascribable to 16 genera, all belonging to the phylum Ascomycota (Table 1). *Aspergillus* and

Penicillium were the most represented genera (34% of the total species and 65% of the total microbial load, 25.5% of the total species and 25% of the total microbial load – respectively). Species belonging to *Aspergillus* genus were mainly members of *A. flavipedes* section (*A. micronesiensis*, *A. poliporycola* and *A. spelaeus* - 18.7% of the *Aspergillus* species and 9.9% of the total microbial load), *A. niger* group (*A. awamori*, *A. foetidus*, *A. niger* and *A. tubingensis* - 31.2% of the *Aspergillus* species, 6.6% of the total microbial load) and *A. versicolor* group (*A. creber*, *A. fructus*, *A. protuberus*, *A. sidowii* and *A. versicolor* - 31.2% of the *Aspergillus* species and 6.4% of the total microbial load).

Other genera recorded with more than one species were *Chaetomium* (3 species), *Acremonium*, and *Trichoderma* (2 species). All the animals were colonized by fungi and statistical analyses did not show significant differences amongst animals (p: 0.213, Figure 1).

Please insert here Figure 1

The fungal species occurrence on different animal specimens highlighted two most frequent species, *Aspergillus awamori* and *Penicillium steckii*, isolated from 5 out of 6 animals. Other species detected with high frequency were *Aspergillus insuetus* (4 animals) *A. fructus*, *A. niger*, *A. tubingensis*, *Penicillium brevicompactum*, *P. citrinum* and *Stachybotrys chartarum* (3 animals).

In regard to the three matrices under analyses (body wall, intestine, faeces), the number of isolated species ranged from 25 associated with the intestine to 22 species isolated from the body wall and faeces. Eighteen of these were isolated from more than one matrix. In terms of global biodiversity, the Shannon

index (H') indicated a similar biodiversity level among the 3 districts. Gini-Simpson dominance index pointed out comparable and high species dominance in all the districts (1-Lambda - Table 2).

Please insert here Table 2

Aspergillus was the dominant genus, representing 65% of the total microbial load detected on the three districts and the only ubiquitous genus with 5 species (*A. awamori*, *A. fructus*, *A. ochraceus*, *A. tubingensis* and *A. versicolor*) detected on all districts. These species colonised with a higher rate faeces (average of 304.5 ± 81.9 CFU gdw⁻¹) and intestine content (268.7 ± 95.7 CFU gdw⁻¹), compared to the body wall (65.5 ± 26.8 CFU gdw⁻¹). *Penicillium* was the other district-related dominant genus (25% of the total microbial load): *P. steckii* mainly colonised the faeces (761.7 ± 71.8 CFU gdw⁻¹) and intestine (267.1 ± 42 CFU gdw⁻¹), *P. citrinum* colonised the same districts with a higher load in the faeces (118.8 ± 17.2 CFU gdw⁻¹) compared to the intestine (11.9 ± 2 CFU gdw⁻¹), and *P. brevicompactum* occurred with a higher load in the faeces (578.1 ± 81.1 CFU gdw⁻¹) than the body wall (15 ± 2.5 CFU gdw⁻¹).

Please insert here Table 1

Fungal extracts chemical analyses

Sixteen fungal strains were grown for chemical extraction following a preliminary OSMAC approach. Fungi were cultured on two media with sodium chloride, one nutrient rich (SMA) and one nutrient poor (MECIA) and on one nutrient rich medium without sodium chloride (MEA). After four weeks at 24°C, fungal biomass and growing medium metabolites were extracted using both

polar and non-polar solvents. Average obtained raw extract dry mass on MEA was 153mg, 335mg on MECIA and 410mg on SMA.

The extracts chemical profiling was performed through HPLC: extracts belonging to nine strains generated different metabolic profiles on the three growing media (four examples in Figure 2). Qualitative changes in their metabolic profiles (i.e. peaks at different retention time in the chromatograms of the three media) were reported for all *Penicillium* strains analysed (*P. brevicompactum* MUT 1097, *P. chrysogenum* MUT 1115, *P. citrinum* MUT 1071, and *P. citrinum* MUT 1105) as well as *Acremonium implicatum* MUT 1055, *Acrostalagmus luteoalbus* MUT 1070, *Chaetomium globosum* MUT 1013, *Chaetomium* sp. MUT 1035, and *Myrotecium verrucaria* MUT 1069. For these species, SMA medium chromatogram showed the highest number of peaks. Variation in the metabolites production based on the culture conditions was recorded for both different species of the same genus (*Aspergillus insuetus* and *A. protuberus*) and for different strains of the same fungal species (*P. citrinum*).

Please insert here Figure 2

Fungal extracts cytotoxicity

The fungal extracts' cytotoxicity was evaluated by exposing HepG2 cells to seven extracts' concentrations (200ng ml⁻¹ - 200µg ml⁻¹) and measuring the viability after 72 h. The established threshold to consider an extract cytotoxic was set at 15% of induced cell death: results showed no extracts' cytotoxicity between 200pg ml⁻¹ and 20ng ml⁻¹; two extracts were cytotoxic at 200ng ml⁻¹;

thee extracts (0.6%) were cytotoxic at 2µg ml⁻¹; 17 (34.4%) at 20µg ml⁻¹ and 46 extracts (95.6%) were cytotoxic at 200µg ml⁻¹. The LD₅₀ recorded (Table 3) ranged from 27.7 to 474.3µg ml⁻¹: 46% of the extracts in the range of 0-100µg ml⁻¹, 27% in the range 100-200µg ml⁻¹, 19% in the range 200-300µg ml⁻¹, 2% in the range 300-400µg ml⁻¹ and 6% in the range 400-500µg ml⁻¹. The most cytotoxic extracts were produced by four species of which two belonged to *Chaetomium*, one *Acrostalagmus* and one *Myrotecium* genera (Figure 3).

The influence of growing medium on fungal synthesis of cytotoxic compounds (OSMAC effect) was also evaluated. Cell death induced by three extracts belonging to each single species were compared using one-way ANOVA statistical test. All 16 strains demonstrated statistically different cytotoxicity of extracts obtained from the three culture media, for at least one extract concentration tested (Figure S1).

Please insert here Figure 3

Please insert here Table 3

Fungal extracts osteogenesis

In order to evaluate the potential of fungal extracts to promote hMSCs osteogenic differentiation, the 48 extracts were tested using an automated HTS platform. Extracts diluted in DMSO were tested at the four highest nontoxic concentrations defined by the preliminary cytotoxic investigation (less than 15% cell death induced). Possible promoters of osteogenesis were detected from this screening selecting values above a threshold: ALP expression higher than positive control average + three times the standard deviation (Figure S2).

Using this method, 9 extracts were selected as osteogenic promoters and re-screened (MUT 1115-SMA; MUT 1071-SMA; MUT 1097-MEA; MUT 1086-SMA; MUT 1074-SMA; MUT 1091-SMA; MUT 1054-MEA; MUT 1069-MEA; MUT 1035-MECIA). These extracts were manually tested using the same procedure and miniaturized assays for ALP and calcium detection. Extracts were added to IOM to evaluate bioactivity as osteogenic inducers, in absence of β -glycerophosphate in the differentiation medium or added to OM to evaluate improvement of marker expression in presence of all the osteogenic differentiation factors. A significant increase in ALP expression after treatment was detected for four extracts when tested in IOM (Figure 4.A), showing to be able to induce differentiation in absence of β -glycerophosphate in the culture medium. When tested in OM (Figure 4.B), two of these extracts showed to promote a significant increase of ALP compared to the positive control cells treated with OM. In terms of calcium mineralization, no extract was able to induce differentiation when tested in IOM (Figure 4.C) while two extracts showed to improve the cell differentiation by increasing the quantity of mineralized calcium when tested in OM (Figure 4.D). Overall, extracts 1115-SMA and 1071-SMA belonging to *Penicillium chrysogenum* and *P. citrinum*, respectively, showed to improve the osteogenic marker expression ALP and calcium mineralization by hMSCs after treatment.

Please insert here Figure 4

Discussion

Our study demonstrates that the Mediterranean marine echinoderm *Holothuria poli* is widely colonized by fungi in its internal and external body sectors.

384 Isolated fungal strains all belonged to the phylum Ascomycota, confirming it as
385 dominant in the marine environment (Jones *et al.* 2019). Our data show a
386 significantly higher fungal diversity (47 species) compared to that reported from
387 previously investigation on the same animal (Omran and Allam 2013) as well
388 as to that reported from other species of sea cucumbers (Pivkin 2000). Omran
389 and Allam (2013) reported only a single isolate, *Candida albicans*, associated
390 with *H. poli* from the Egyptian Mediterranean Sea, a species not detected in our
391 study. Pivkin (2000) studied sea cucumbers from the Pacific Ocean, reporting
392 25 fungal species from *Eupentacta fraudatrix*, nine from *Apostichopus*
393 *japonicus* and three from *Cucumaria japonica*. The higher diversity that we
394 reported can probably be related to the isolation techniques adopted compared
395 to the previous studies: Pivkin (2000) used agar with holothurian broth, with
396 NaOH added; Omran and Allam (2013) used Sabouraud and blood agar media
397 without the addition of salt. Our isolation strategy implemented the use of CMA,
398 a nutrient rich medium previously reported as optimal for marine fungi isolation
399 (Kossuga *et al.* 2011), added of sea salt to mimic the environmental conditions.
400 In terms of species recurrence, Mediterranean *H.poli* showed four fungal
401 species in common to the Pacific sea cucumber isolated by Pivkin (2000):
402 *Alternaria alternata*, *A. versicolor*, *C. sphaerospermum* and *Penicillium*
403 *commune*. In comparison with the mycobiota isolated from other Mediterranean
404 substrates *H. poli* showed more similarities (Fig. 5): ten species in common with
405 the brown algae *Padina pavonica* (Garzoli *et al.* 2018), nine in common with
406 the green algae *Flabellia petiolata* (Gnavi *et al.* 2017), nine with the seagrass
407 *Posidonia oceanica* (Panno *et al.* 2013), eight with the sponge *Psammocinia*
408 sp. (Paz *et al.* 2010) and five with decaying woods (Garzoli *et al.* 2015). Most

common isolated fungal species from Mediterranean marine substrates are *T. harzianum* and *P. brevicompactum*, respectively isolated in the past from Oceanic sponges and ascidians (Yamada *et al.* 2014; Vacondio *et al.* 2015) and from Oceanic algae and sponges (Alves 2019; Bovio *et al.* 2019). Such common species are likely to be highly adapted to the marine environment showing a high potential in substrate colonization, therefore their specific role in association with animals, plants and algae should be thoroughly investigated to identify possible key marine species.

Please insert here Figure 5

Eleven species isolated in this study represent new records for the marine environment worldwide: *A. creber*, *A. foetidus*, *A. fructus*, *A. micronesiensis*, *A. spelaeus*, *Auxarthron ostraviense*, *Chaetomium subaffine*, *Emericella quadrilineata*, *Myriodontium. keratinophilum*, *P. adametzii*, and *Trichoderma epimyces*. Several of them have been previously described as soil inhabitants (Pitt 1979; Doveri *et al.* 2013; López-Quintero *et al.* 2013; Nováková *et al.* 2014) or present in house dust (Samson *et al.* 2014a). *Auxarthron ostraviense*, *M. keratinophilum* and *E. quadrilineata* are usually reported from soils and are described as weak human pathogens (Maran *et al.* 1985; Gugnani *et al.* 2007; Hubka *et al.* 2013). Further records on new substrates are needed to elucidate their presence in the marine environment.

The isolated fungal community was characterized by the dominance of *Penicillium* and *Aspergillus* genera, as previously detected on other Mediterranean substrates (Paz *et al.* 2010; Panno *et al.* 2013). Species belonging to these genera are highly sporulating and able to adapt to extremely

disparate environmental conditions such as Antarctica, Atacama desert or deep seas (Godinho *et al.* 2015; Nagano *et al.* 2017; Santiago *et al.* 2018), and therefore are likely to survive in shallow waters and colonize sessile echinoderms. The presence of recurrent species such as *A. awamori* and *P. steckii* suggests a higher adaptation of these taxa to live associated to an animal producing antifungal molecules and a possible selection of the mycobiota operated by the animal.

Our study confirms the pharmaceutical bioactivity of small molecule extracts obtained by fungal strains associated to *H. poli*. Cytotoxicity assessment against hepatocarcinoma cells (HepG2) showed the ability of several strains to produce highly cytotoxic metabolites, a concentration-related bioactivity and the medium influence in the biosynthesis of these compounds. Producers of the most cytotoxic extracts were members of the *Chaetomium* genus, previously reported to synthesize several azaphilones, molecules produced by *Chaetomium globosum* with selective cytotoxicity against leukemia HL60, leukemia L1210 and KB epidermoid carcinoma cell lines (Yamada *et al.* 2012). The marine fungi ability to synthesize human cells' cytotoxic compounds clearly show their potential for the treatment of cancer-based diseases. Future analyses on cytotoxic extracts will involve the investigation of bioactivity retention against other cancer cell lines and the extracts' chemical investigation to purify the molecules responsible of this bioactivity.

Regarding the fungal extracts' bioactivity toward human Mesenchymal Stem Cells, our initial high throughput screening showed nine extracts able to induce differentiation of hMSCs into bone cell progenitors. The positive hits validation was performed by re-testing the extracts and evaluating their specific bioactivity

as inducers or promoters of osteogenic differentiation. This was done by testing the extracts diluted in complete osteogenic medium containing the full cocktail of pro-osteogenic molecules for *in-vitro* differentiation or in incomplete osteogenic medium lacking β -glycerophosphate. Cells differentiation after treatment was evaluated by measuring two osteogenic markers: intracellular alkaline phosphatase production and cells calcium mineralization. Extracts obtained by *P. chrysogenum* (MUT 1115) and *P. citrinum* (MUT 1071) induced and promoted osteogenic differentiation as showed by increased level of intracellular ALP and increased amount of calcium mineralized in the extracellular matrix, process leading to bone formation, demonstrating great potential for the discovery of new drug candidates for regenerative medicine. While this is the first record for *P. chrysogenum* producing compounds with pro-osteogenic bioactivity, *P. citrinum* was previously reported to synthesize mevastatin (Endo *et al.* 1976), a molecule belonging to a class of metabolites, statins, known for their bioactivity as cholesterol-lowering and documented to stimulate the formation of new bone tissue (Mundy *et al.* 1999; Morse *et al.* 2018).

The culture conditions' influence on fungal metabolite production (OSMAC effect) was demonstrated both with chemical profiling and detected extracts bioactivity. The variable cytotoxic bioactivity of extracts belonging to the same strain growing on different media was confirmed by the extracts chemical analyses that showed chromatograms variability. Most effective medium for the biosynthesis of bioactive compounds was SMA, responsible for the production of the most cytotoxic extracts in terms of number and induction of cell death, and the main medium where osteogenic metabolites were synthesized. Once

again is demonstrated that the discovery of new fungal secondary metabolites for biotechnological applications is strictly linked to the modification of culture conditions to trigger different metabolic pathways and induce the production of the widest fungal metabolome.

In this study we prove as demonstrated before (Lagarde *et al.* 2018) the fungal intraspecific variation in the production of bioactive compounds: different strains of *P. citrinum* isolated in this study showed different chromatographic profiles and different detected bioactivity as cytotoxicity and pro-osteogenic compounds. This evidence highlights the importance of strains evaluation for the discovery of new compounds for specific pharmaceutical applications in order to widen the chances to discover strains with pharmaceutical potential.

To the best of our knowledge, this is the first record on marine fungal extracts inducing hMSCs osteogenic differentiation in comparison to the established inducer, β -glycerophosphate, and potential producers of new molecules for regenerative medicine. Positive extracts will therefore be further analysed to identify the responsible molecules associated with the detected bioactivity and describe potential drug candidates. Further evidences of cytotoxic selectivity and hMSC osteogenic induction by the metabolites produced by our strains are needed to evaluate progression towards their use in targeted cell therapies, particularly in diseases such as cancer (Cragg *et al.* 2009), osteoporosis and osteogenesis imperfecta (Grunevald *et al.* 2014; Besio and Forlino 2015, Gagliardi *et al.* 2017). Furthermore, the optimized High Throughput assays developed in the present study allowed us to test our extracts library in a time and cost effective way and are a valuable tool to foster studies of cell-related bioactivity from wide natural compounds libraries.

To conclude, a peculiar fungal community with an interesting metabolic potential was recorded on *Holothuria poli*, increasing the knowledge about animal fungal association in the Mediterranean Sea, a marine biodiversity hotspot whose microbial community is substantially underestimated. The importance of culture based fungal community investigation in biodiscovery research was once again underlined: the development of future studies to unlock fungal biotechnological potential is strictly connected to the conservation of pure strains in culture.

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Conflict of Interest:

The authors have no conflict of interest to declare

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Table 1: Marine fungal species recovered from *Holothuria polii* in the Tunisian Sea

Taxa	MUT code	Animal*						Matrix*		
		1	2	3	4	5	6	B	I	S
<i>Acremonium alternatum</i> Link 1809	1054						17.02		17.02	
<i>Acremonium implicatum</i> (J.C. Gilman & E.V. Abbott) W. Gams 1975	1055				12.82			12.82		
<i>Acrostalagmus luteoalbus</i> Pethybr. 1919	1070 2130		14.95	21.22				14.95	21.22	
<i>Alternaria alternata</i> (Fr.) Keissl. 1912	856			63.66					63.66	
<i>Aspergillus awamori</i> Nakaz. 1907	2012 2060 2099 2191	86.29	384.6	487.8		163.46	17.02	86.29	483.90	569.08
<i>Aspergillus creber</i> Jurjevic, S.W. Peterson & B.W. Horn 2012	1999 2026		53.41					14.95		38.46
<i>Aspergillus foetidus</i> Thom & Raper 1945	2035					100.59				100.59
<i>Aspergillus fructus</i> Jurjevic, S.W. Peterson & B.W. Horn 2012	2175 2187 2200 2203	44.86	130.34	466.88				32.21	466.88	142.92
<i>Aspergillus insuetus</i> (Bainier) Thom & Church 1929	1085 2054 2092	15.47	29.91	21.22		24.74		42.16	49.18	
<i>Aspergillus micronesiensis</i> Visagie, Hirooka & Samson 2014	1990 1992	34.51	59.82					94.34		
<i>Aspergillus nidulans</i> (Eidam) G. Winter 1884	2165			21.22					21.22	
<i>Aspergillus niger</i> Tiegh. 1867	2082		1692.3	41.99			87.24			1821.5
<i>Aspergillus ochraceus</i> G. Wilh. 1877	2036 2086 2096 2105		680.06	122.79				161.4	295.3	346.15
<i>Aspergillus polyporicola</i> Hubka, A. Nováková, M. Kolarík & S.W. Peterson 2015	1456					528.12				528.12
<i>Aspergillus protuberus</i> Munt.-Cvetk. 1968	1091					24.97			24.97	
<i>Aspergillus pseudodeflectus</i> Samson & Mouch. 1975	2040		38.46							38.46
<i>Aspergillus</i> sp.	2106			21.22		12.48			33.70	
<i>Aspergillus spelaeus</i> A. Nováková, Hubka, M. Kolarík & S.W. Peterson 2015	1457 1993					352.08	17.02		17.02	352.08
<i>Aspergillus sydowii</i> (Bainier & Sartory) Thom & Church 1926	2072 2102		14.95			12.48		14.95	12.48	
<i>Aspergillus tubingensis</i> Mosseray 1934	1074 2043 2047	41.41	76.92				67.06	33.01	34.04	118.33
<i>Aspergillus versicolor</i> (Vuill.) Tirab. 1908	1086 2052 2190		361.11	63.66				14.95	63.66	346.15

<i>Aureobasidium pullulans</i> (de Bary & Löwenthal) G. Arnaud 1918	2186				12.48				12.48	
<i>Auxarthron ostraviense</i> Hubka, Dobíášová & M. Kolařík 2012	1556	14.95						14.95		
<i>Cadophora luteo-olivacea</i> (J.F.H. Beyma) T.C. Harr. & McNew 2003	1073		31.49						31.49	
<i>Chaetomium globosum</i> Kunze 1817	1013	14.95						14.95		
<i>Chaetomium</i> sp.	1035		21.22						21.22	
<i>Chaetomium subaffine</i> Sergeeva 1961	1015		21.22			17.02			38.24	
<i>Cladosporium sphaerospermum</i> Penz. 1882	1454	27.60								27.6
<i>Emericella quadrilineata</i> (Thom & Raper) C.R. Benj. 1955	2019	271.28	21.22					44.87	247.63	
<i>Myrothecium verrucaria</i> (Alb. & Schwein.) Ditmar 1813	1069	14.95						14.95		
<i>Myriodontium keratinophilum</i> Samson & Polon. 1978	1443			12.82	12.48			12.82	12.48	
<i>Paecilomyces lilacinus</i> (Thom) Samson 1974	992	29.91						29.91		
<i>Penicillium adametzii</i> K.M. Zaleski 1927	2107	38.46								38.46
<i>Penicillium antarcticum</i> A.D. Hocking & C.F. McRae 1999	2125	41.41				17.02			17.02	41.41
<i>Penicillium brevicompactum</i> Dierckx 1901	1097									
	2061	514.95		27.83	50.29			14.95		578.13
	2097									
	2140									
<i>Penicillium chrysogenum</i> Thom 1910	1115	29.91						29.91		
<i>Penicillium citreonigrum</i> Dierckx 1901	2062	269.23								269.23
<i>Penicillium citrinum</i> Thom 1910	1071	13.80	11.91	104.99					11.91	118.79
<i>Penicillium commune</i> Thom 1910	1105									
	2120	15.47						15.47		
<i>Penicillium corylophilum</i> Dierckx 1901	2173	69.01								69.01
<i>Penicillium oxalicum</i> Currie & Thom 1915	2094		44.87	42.44				44.87	42.44	
<i>Penicillium roseopurpureum</i> Dierckx 1901	2149							86.29		
	2146	86.29								
<i>Penicillium steckii</i> K.M. Zaleski 1927	1453		423.07	317.65	13.91	12.48	261.73		267.14	761.72
	2109									
<i>Pleosporales</i> sp.	2081	38.46								38.46
<i>Stachybotrys chartarum</i> (Ehrenb.) S. Hughes 1958	1554	13.80	38.46		12.82			12.82		52.26
<i>Trichoderma epimyces</i> Jaklitsch 2008	1555					12.48			12.48	
<i>Trichoderma harzianum</i> Rifai 1969	2022									
	2009	192.3								192.3
Mean animal fungal load (CFU/gdw)		40.83	203.1	111.3	16.05	101.5	62.65	38.34	92.12	287.9
Number of detected species		12	27	17	5	13	8	22	25	22

*species detected in each animal (1 to 6) and matrix (B= Body wall; I= Intestine; S= Faeces)

Table 2: Biodiversity within districts:
Shannon-Weaver's index (H') and Gini-
Simpson's index (1-Lambda)

Matrix	N° Species	H'	1-Lambda
B	22	3.007	0.954
I	25	3.071	0.946
S	22	3.013	0.944

B=Body wall; I=Intestine; S=Faeces

Table 3 Cytotoxicity of crude extracts on human hepatocarcinoma cell line (HepG2)

Species	MUT code	Growing medium		
		SMA	MECIA	MEA
<i>Acremonium alternatum</i>	1054	>200	>100	>200
<i>Acremonium implicatum</i>	1055	>200	>200	>200
<i>Aspergillus insuetus</i>	1085	>200	>100	62.1
<i>Aspergillus protuberus</i>	1091	93.6	>100	71.3
<i>Aspergillus tubingensis</i>	1074	>100	>100	>100
<i>Aspergillus versicolor</i>	1086	82.7	>400	70.4
<i>Cadophora luteo-olivacea</i>	1073	>100	>300	>400
<i>Chaetomium globosum</i>	1013	37.4	60.0	56.3
<i>Chaetomium</i> sp.	1035	49.7	>100	49.4
<i>Chaetomium subaffine</i>	1015	>100	>200	>200
<i>Myrothecium verrucaria</i>	1069	54.5	39.5	27.7
<i>Nectria inventa</i>	1070	41.9	74.3	53.0
<i>Penicillium brevicompactum</i>	1097	80.4	>100	55.0
<i>Penicillium chrysogenum</i>	1115	>100	60.9	70.8
<i>Penicillium citrinum</i>	1071	>100	>400	91.4
<i>Penicillium citrinum</i>	1105	>100	>200	82.7

Cells were treated for 72 hours with increasing concentration of crude extract (from 0.2ng ml⁻¹ to 0.2mg ml⁻¹) or controls. Values are calculated as LD₅₀ in µg ml⁻¹

Figure Captions

Figure 1. PCA analyses of animals and districts. Triangle pointing upward is Body wall (B); triangle pointing downward is Intestine (I); square is Sand (S).

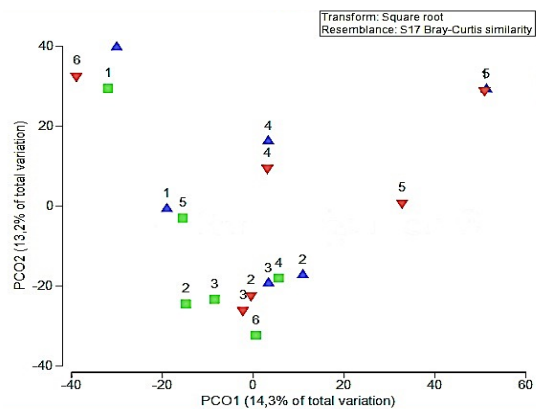
Figure 2. Fungal extracts HPLC chromatographic profiles at 280nm. Crude extracts obtained by fungal growth on different media (Soy Mannitol Agar, SMA; Malt Extract Chloride Agar, MECIA; Malt extract Agar, MEA) were analyzed by HPLC to detect variability in the metabolites content. In the figure are showed the extracts profiles on the different growing media of two strains belonging to the same genera (*Aspergillus insuetus*, *Aspergillus protuberus*) and two different strains of the same species (*Penicillium citrinum*).

Figure 3. Fungal extracts cytotoxicity toward hepatocarcinoma (HepG2) cells. The extracts' cytotoxicity of four of 16 fungal strains, encompassing the most cytotoxic, are here showed. Five thousand cells were seeded in 96-well plates and treated with fungal extracts for 72 hours at 37°C, 5% CO₂. Extracts dissolved in DMSO were diluted in cell growing medium to reach seven increasing concentrations to treat the cells. Positive control cells (ctr+, dashed line) were treated with growing medium +0.5% DMSO while negative control cells (ctr-, dotted line) were treated with growing medium +10% DMSO. Cell viability after treatment was measured by adding 10% v/v of resazurin based dye (alamarBlue) and incubating at 37°C, 5% CO₂ for 6 hours. Fluorescence was then read at 531nm excitation wavelength, 572nm emission wavelength. Light grey line with round dot is extract from MEA medium; dark grey line with triangle dot is extract from MECLA medium; black line with rhomboidal dot is extract from SMA medium.

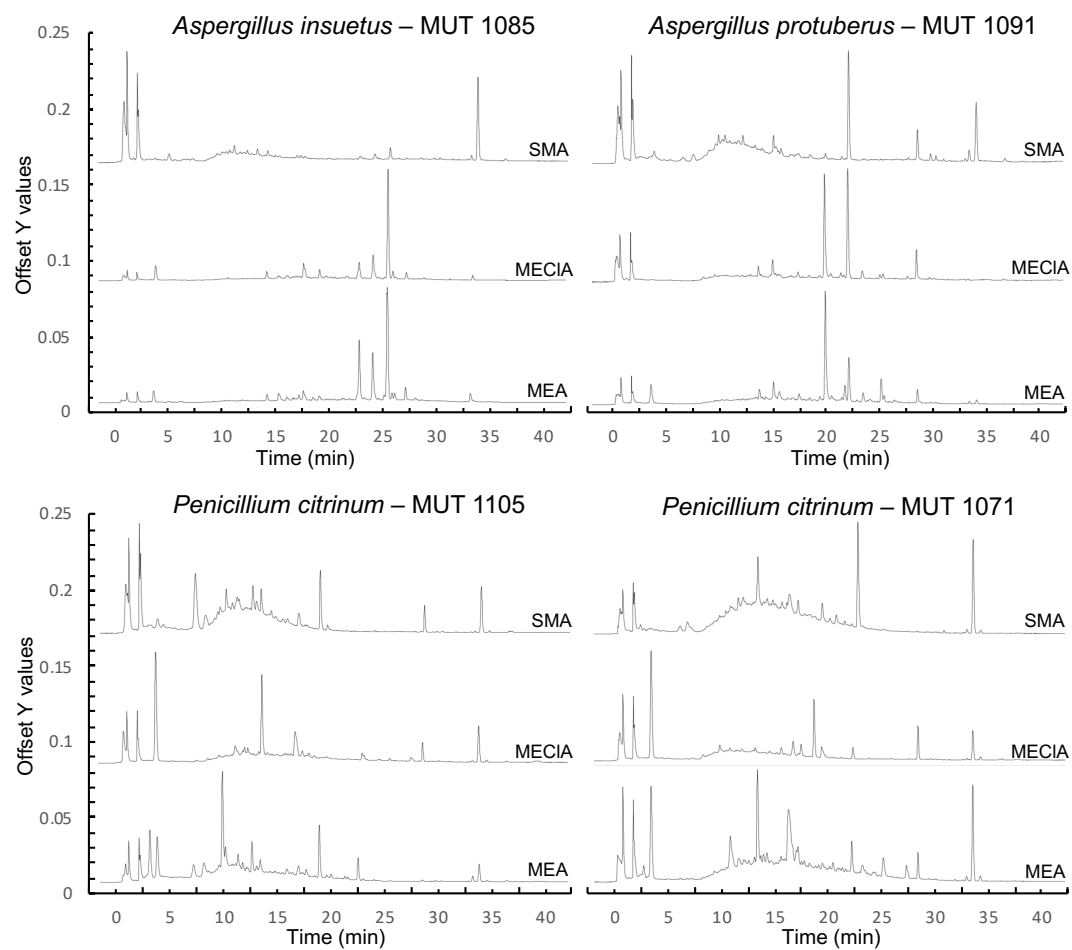
Figure 4. Positive hit extracts re-tested for osteogenic bioactivity. hMSCs differentiation after fungal extracts treatment was evaluated by measuring two markers. Early differentiation marker alkaline phosphatase (ALP) expression was measured 7 days after treatment. Cells were exposed to 20µg ml⁻¹ fungal extract dissolved in Incomplete Osteogenic medium to evaluate their ability to induce differentiation in absence of β-glycerophosphate in the medium (A) or in Osteogenic Medium to evaluate an improvement on the differentiation induced by the standard differentiation medium (B). Late differentiation marker, matrix mineralized calcium, was measured 12 days after treatment. Cells were treated with extracts dissolved in IOM (C) or OM (D). The experiment was carried out on experimental triplicates. Results are presented as the mean ± SD of 3 technical replicates, * = p ≤ 0.05 calculated using ANOVA one-way with Bonferroni post-test.

Figure 5. Fungal species occurrence on Mediterranean substrates. The Venn diagram highlights common fungal species between *Holothuria poli* and other Mediterranean substrates.

780 **Figures**



781 **Figure 1**
782



783 **Figure 2**
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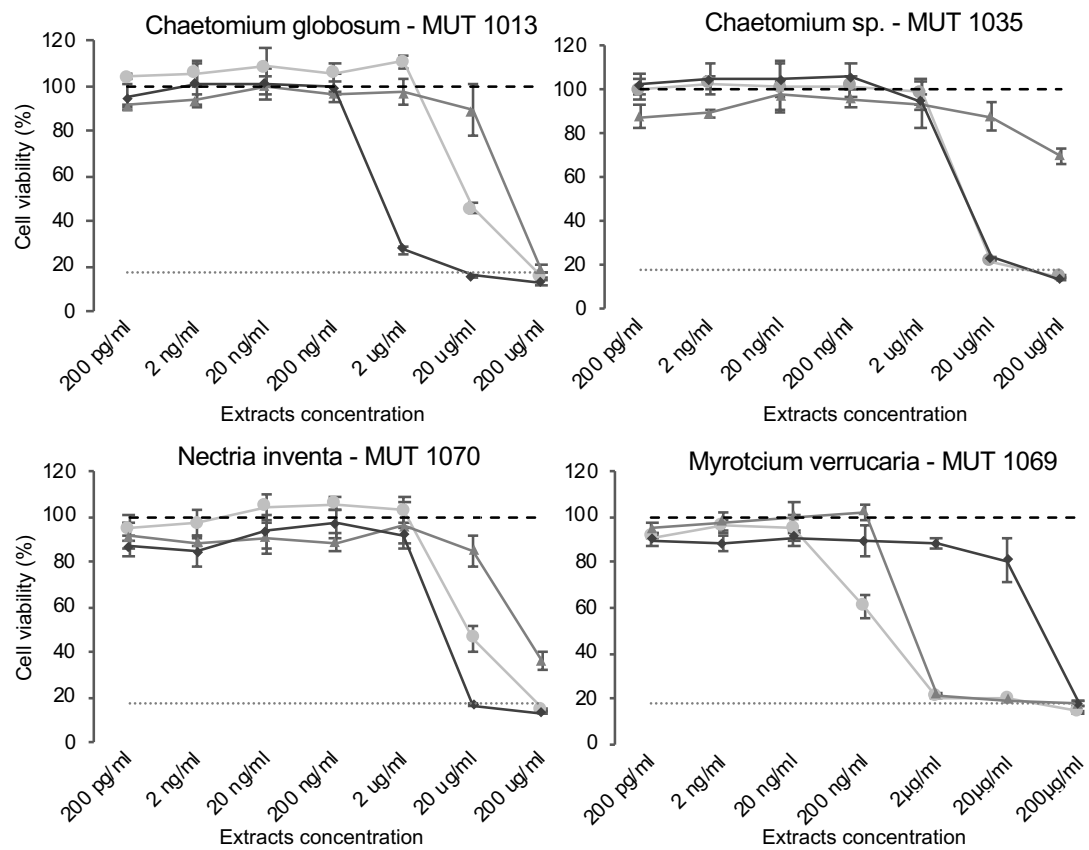


Figure 3

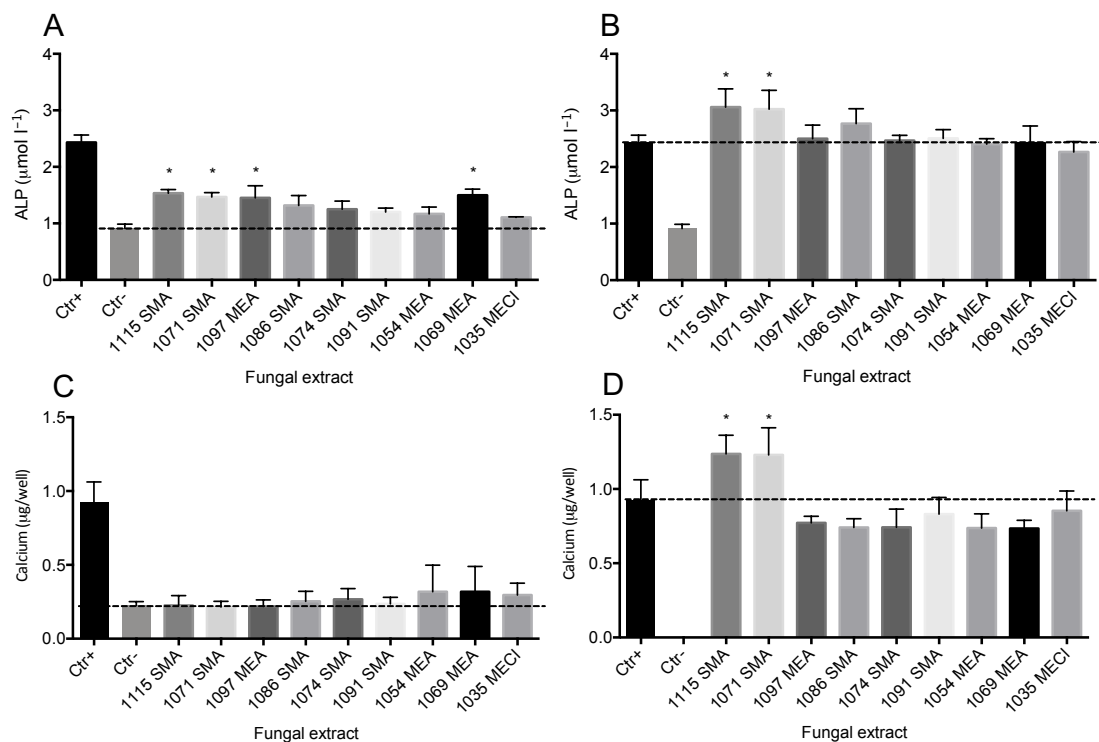


Figure 4

Holothuria poli

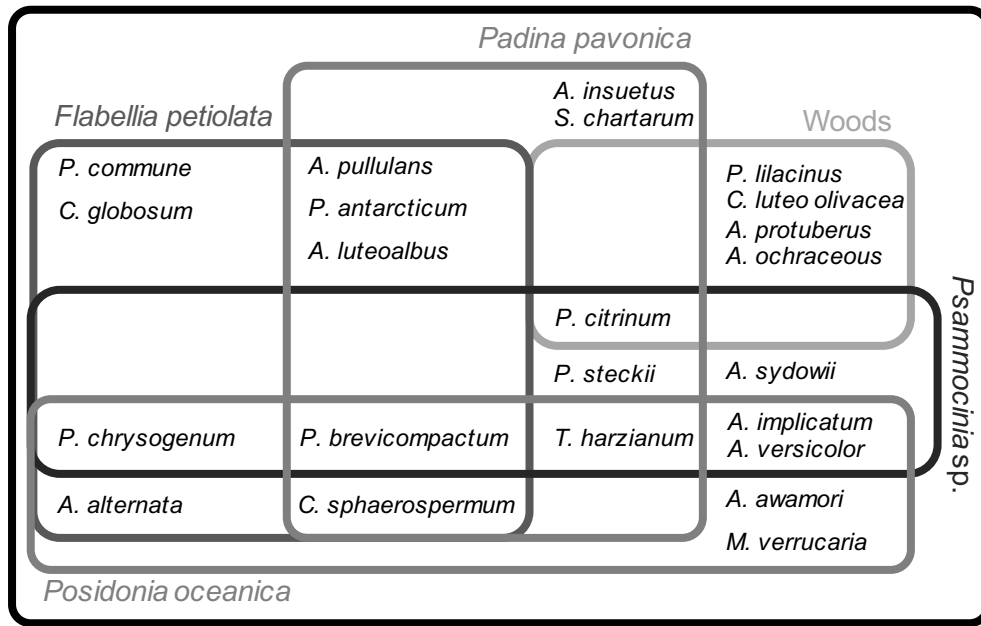


Figure 5